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on

METHODS AND COMPOSITIONS FOR ANALYZING FOR SERINE HYDROLASES

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METHODS AND COMPOSITIONS FOR ANALYZING FOR SERINE HYDROLASES

INTRODUCTION

Technical Field

The field of this invention is assaying for serine hydrolases.

Background

With the post-genome era rapidly approaching, new strategies for the functional analysis of proteins are needed. Most conventional approaches focus on recording variations in protein level rather than activity. The ability to measure the activity of a protein would greatly accelerate an understanding of the protein's function.

The advent of proteomics has opened up new avenues for studying proteins and the manner in which they interact. Serine proteases play important roles in numerous developmental and tissue-specific events *in vivo*, including blood coagulation, inflammation, angiogenesis, neural plasticity, peptide hormone processing and T-lymphocyte-mediated cytotoxicity. Additionally, several human diseases are associated with dysfunctions in serine proteases and/or their endogenous inhibitory proteins, including hemorrhagic disorders, emphysema, and cancer.

The large number of mammalian serine hydrolases identified to date is both impressive and perplexing, with the endogenous functions of many members of this enzyme family remaining unknown. As ORF's encoding putative serine hydrolases continue accumulate in public databases, the need for alternative experimental methods to study these enzymes is evident. There is both an interest in having specific ways to identify specific serine hydrolases and ways that allow for identifying groups of serine hydrolases.

Potent serine hydrolase inhibitors include fluorophosphonate/fluorophosphate derivatives, such as diisopropyl fluorophosphate, while enzymes such as the cysteine, aspartyl, and metallohydrolases are for the most part relatively inert to such agents. It is

known that for the fluorophosphonate/fluorophosphate derivatives to be active, the enzyme must be catalytically active. It is, therefore, of interest to find agents that can measure the presence of catalytically active serine hydrolases.

Related Art

Creighton, T. E. (1993) *Proteins: structure and Molecular Properties* (Freeman, New York), 2nd Ed. and Walsh, C.T. (1979) *Enzymatic Reaction Mechanisms* (Freeman, New York), teach the use of dialkyl fluorophosphonates as serine hydrolase inhibitors. Liu, et al. (1999) PNAS USA 96:14694-14699 discloses the use of fluorophosphonate derivatives for assaying serine hydrolases. See also, the references cited therein. Patricelli and Cravatt (1999) Biochemistry 38:14125-14130, describes the activity of fatty acid amide hydrolase herein incorporated by reference in its entirety.

SUMMARY OF THE INVENTION

Novel conjugated fluorophosphoric acid derivatives are provided for the selective inhibition of serine hydrolases. The derivatives are found to inhibit catalytically active enyzmes irreversibly and the rate of inhibition varies with the particular serine hydrolase, so as to allow for differentiation between different hydrolases, based on their inhibition profile. The inhibition of serine hydrolase activity may be used in diagnostics for differentiating between normal and abnormal cellular function, correlating serine hydrolase activity with cellular status and isolating and identifying serine hydrolases present in a sample, such as a cellular lysate.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention compositions and methods are provided for quantitatively identifying one or more serine hydrolases present in a sample, particularly a complex mixture of proteins and as affinity agents for isolation and

determination of members of the serine hydrolase family. The compounds employed are derivatives of alkyl-substituted fluorophosphoric acids, particularly fluorophosphonates and fluorophosphates.

For the most part the compounds come within the following formula:

$$X - L - R - (O)_m - P(O)(OT) - F$$

wherein:

F, P and O have their normal meaning of fluoro, phospho and oxy;

X is a ligand or detectable label;

L is a linking group;

R is an aliphatic group of at least 2 carbon atoms, usually at least 4 carbon atoms and not more than about 16 carbon atoms, usually not more than about 12 carbon atoms, usually being straight chain alkylene, saturated or unsaturated, usually having not more than 2 sites of unsaturation;

m is 0 or 1; and

T is alkyl of from 1 to 6, usually 1 to 3 carbon atoms.

For the most part the ligand will be biotin and the detectable label will be a fluorescent compound, e.g. fluorescein, rhodamine, Texas red, etc.

Of particular interest are compounds where the linking group includes a dicarboxamido-α,Ω-alkylene, particularly where biotin is used which includes a

carboxyl group naturally. The alkylene will generally be of about 2 – 6 carbon atoms, the length will be desirably related to the length of the R group, so as to provide a total chain length between the phosphoric acid group and the ligand that does not interfere with the activity of the phosphoric acid group, nor the binding of the ligand to its respective receptor.

The ligand can be any ligand that does not interfere with the binding of the subject compounds to the serine hydrolases, relatively small, less than about 1kDal, frequently less than about 500Dal, has an appropriate receptor and is synthetically accessible. There are a number of popular ligands, such as biotin, dethiobiotin, digoxin and derivatives thereof, fluorescein, etc. These ligands have strongly binding natural receptors, such as avidin and streptavidin ("strept/avidin") for biotin and dethiobiotin, and antibodies for the remaining listed ligands. In some instances it will be desirable to release the serine hydrolase bonded to the inhibitor of this invention. A useful pair is dethiobiotin, which can be replaced by biotin.

The subject compounds can be prepared using an α, Ω-(halo or pseudohalo)alkene, where the halo or pseudohalo group is displaced with a trialkylphosphite, followed by selective oxidation of the olefin to a carboxy or aldehyde. The activated carboxy, e.g. N-succinimidyl ester or carbodiimide anhydride, may be reacted with the ligand or detectable label bonded to a linking group terminating in an amino group to form an amide. The aldehyde may be bonded to an amine by forming an imine or Schiff's base or by reductive amination, forming an alkylated amine. Other than the ligand or the label, the subject compounds will have fewer than 30 carbon atoms, usually fewer than 25 carbon atoms. There will be 1 or more functionalities in the chain joining the ligand or detectable label to the alkyfluorolphosphoric acid group, generally neutral functionalities, such as amido, oxy, thio, urea, thiourea, etc.

When the subject compound is bound to the serine hydroxyl of a serine hydrolase, the resulting inhibited enzyme will have the following formula:

where all of the symbols have been defined previously, except for SH, which is a serine hydrolase bonded at the serine group of the active site to the phosphate.

Known serine hydrolases include fatty acid amide hydrolases (FAAH), kallikreins, acylpeptide hydrolases, prostate specific antigen, cholinesterases, trypsins, chymotrypsins, plasmin, thrombin, phospholipases, signal peptidases, amidase signature enzymes, plasminogen activators, prohormone convertases, granzymes, seprase, dipeptidyl and tripeptidyl peptidases, usually being derived from mammalian sources, particularly human, but may come from other sources, both prokaryotic and eukaryotic, including plants, birds, invertebrates, fungi, etc.

The subject inhibitors may be used in a variety of ways. One application is to determine the serine hydrolase activity of a physiological sample. The sample may be blood, cells, tissue, or other physiological sample of interest. In some situations, samples that are suspected of having one or have serine hydrolases may be monitored, as in the genetic engineering of serine hydrolase proteins, where the efficiency of synthesis would be of interest. In the case of tissue or cells, the cells may be lysed in accordance with conventional conditions, using a homogenizer, blender, pellets, centrifuge or other convenient device. The resulting lysed cellular composition may be centrifuged and the supernatant adjusted for protein content. Depending on the nature of the ligand, the supernatant fraction may be freed of naturally occurring ligand and/or receptor. The supernatant may be further treated, as appropriate, adding buffer, further dilution, fractionation by chromatography, etc. Where fractionated, individual fractions will be used in the assay.

Candidate compounds to be used as therapeutics associated with indications involving serine hydrolase dysfunction, particularly for inhibiting specific or groups of serine hydrolases, may be monitored by preparing a reaction mixture with one or more hydrolases and monitoring the effect on the rate of inhibition. One would add one or more subject compounds and a candidate compound and then monitor the rate of

inhibition, by isolating aliquots and analyzing the aliquot for serine hydrolase activity or isolating bound serine hydrolases and analyzing the bound serine hydrolases.

The sample will generally be adjusted to have between about 0.1 to 5 μg/ml, more usually 0.5 to 2 μg/ml of protein, larger amounts being unnecessary. The sample may be added in conventional buffers, such as Tris, HEPES, phosphate, etc. Most chemically and biologically inert buffers providing the desired pH may be used. The fluorophosphoric acid compound can be combined dry or preferably as a water-miscible polar organic solution that is chemically and biologically inert under the conditions of the assay. Ethanol, DMSO, DMF, etc. may be used. Usually, an excess of the fluorophosphoric acid compound will be employed, generally at least about 2 fold excess, usually at least about 5 fold excess, based on actual serine hydrolase concentration, if known, or estimated serine hydrolase, based on total protein, to ensure that substantially all of the available serine hydrolase has reacted. The mixture will ordinarily be incubated for about 10min or more, usually not more than about 1h. The reaction may then be quenched with any convenient quenching agent, particularly elevated temperatures. The resulting denatured composition may then be analyzed, for example, using capillary electrophoresis (microfluidic device), gel electrophoresis, HPLC, mass spectrometry (MALDI), Western blotting, or the like, where the fractions may be observed by virtue of the ligand. Either the ligand provides a detectable signal, usually fluorescence, although electrochemical and chemiluminescence, may also be employed or the ligand may be reacted with a labeled receptor, and detected as the ligand-receptor complex. The receptor may be conjugated with an enzyme that produces a colored or fluorescent product. There are many enzymes known for this purpose. Illustrative enzymes include horse radish peroxidase, β-galactosidase, G6PDH, MDH, alkaline phosphatase, lysozyme, etc. In many cases commercially available substrates can be used for detection.

The subject compositions may be used for diagnosis by determining serine hydrolase activity and/or rate profile of a cellular sample as related to cells in a different state, tissues from different organs, comparing neoplastic or hyperplasic cells to normal cells, activated as distinct from quiescent cells, benign as compared to malignant tumor

cells, cells of different species, etc. Where a disease indication, such as mutations, aberrant metabolism, or the like, is associated with serine hydrolase activity, the serine hydrolase activity can be determined from a source suspected of having the indication. By comparing the result with a known standard, e.g. a standard from a source with the indication or a normal source or both, the subject composition may be used as a diagnostic.

As indicated, the proteins conjugated to the subject compounds may be separated by electrophoresis or other technique, which provides for independent fractions. One can do time courses, to see how the amount of the different fractions changes with time by quantitating the signal. One can also determine the specific serine hydrolases by using antibodies specific for one or more epitopes of each of the serine hydrolases of interest. After separating the protein into distinct bands, the electrophoretic bands may be reacted with labeled antibodies, where the labels are different from the labels of the subject compounds, so as provide a distinguishable signal, e.g. fluorescence at a different wavelength, electrochemical detection as compared to fluorescence, chemiluminescence, etc.

The subject compounds may also be used to identify new serine hydrolases and use the identified serine hydrolases for production of antisera and monoclonal antibodies. For example, the biotinylated serine hydrolases can be isolated and characterized by their mobility, mass fragmenting pattern, etc., in a variety of methods which allow for identification and comparison with known proteins. When the serine hydrolase appears to be a unknown serine hydrolase, it may be sequenced and/or used as an immunogen. The resulting antisera and monoclonal antibodies can be used to compare the epitopes of the newly discovered serine hydrolase with known hydrolases, to screen cellular sources of serine hydrolases for the presence of the newly discovered serine hydrolase and to determine the function of the newly discovered serine hydrolase.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Chemical Synthesis of FP-Biotin. Compound numbers in bold refer to structures shown in Figure 5.

1-[(*p*-Toluenesulfonyl)oxy]-10-undecene (**2**). A solution of **1** (2.0 g, 11.8 mmol, 1.0 equiv) in pyridine (14.0 mL, 177 mmol, 15 equiv) was cooled to 0 °C and treated with *p*TsCl (4.5 g, 23.6 mmol, 2.0 equiv). The reaction mixture was kept at 0 °C for 10 h and then partitioned between ethyl acetate (200 mL) and water (200 mL). The organic layer was washed with 10% aqueous HCl (2 x 200 mL), saturated aqueous NaCl (200 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Chromatography (SiO_2, 5 x 15 cm, 2% ethyl acetate-hexanes) afforded **2** (3.6 g, 3.8 g theoretical, 94%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 7.76 (d, J = 6.5 Hz, 2H, ArH), 7.32 (d, J = 7.3 Hz, 2H, ArH), 5.95-5.75 (m, 1H, RC*H*=CH_2), 5.03-4.90 (m, 2H, RCH=C*H*$_2$), 3.98 (t, J = 6.5 Hz, 2H, CH_2OTs), 2.42 (s, 3H, $ArCH_3$), 2.02 (m, 2H, C*H*$_2$CH=CH_2), 1.65 (p, J = 6.9 Hz, 2H, C*H*$_2$$CH_2OTs$), 1.50-1.20 (m, 12H); MALDI-FTMS (DHB) *m/z* 347.1657 ($C_{18}H_{28}O_3S$ + Na^+ requires 347.1658).

1-Iodo-10-undecene (**3**). A solution of **2** (3.4 g, 10.5 mmol, 1.0 equiv) in acetone (21 mL, 0.5 M) was treated with NaI (3.2 g, 21 mmol, 2.0 equiv) and the reaction mixture was stirred at reflux for 2 h, producing a yellow-orange solution. The reaction mixture was then partitioned between ethyl acetate (200 mL) and water (200 mL). The organic layer was washed sequentially with saturated aqueous $Na_2S_2O_3$ (100 mL) and saturated aqueous NaCl (100 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Chromatography (SiO_2, 5 x 15 cm, 1-2% ethyl acetate-hexanes) afforded **3** (2.3 g, 2.9 g theoretical, 78%) as a colorless oil: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 5.95-5.75 (m, 1H, RC*H*=CH_2), 5.03-4.90 (m, 2H, RCH=C*H*$_2$), 3.16 (t, J = 7.0 Hz, 2H, CH_2I), 2.02 (m, 2H, C*H*$_2$CH=CH_2), 1.80 (p, J = 6.9 Hz, 2H, C*H*$_2$$CH_2I$), 1.50-1.20 (m, 12H).

1-[Bis(ethoxy)phosphinyl]-10-undecene (4). Triethylphosphite (12.2 mL, 71 mmol, 10 equiv) was added to 3 (2.0 g, 7.1 mmol, 1.0 equiv) and the mixture was stirred at reflux for 15 h. The excess triethylphosphite was removed by distillation and the remaining residue submitted to flash chromatography (SiO_2, 5 x 15 cm, 25-50% ethyl acetate-hexanes gradient elution) to afford 4 (1.30 g, 2.1 g theoretical, 62%) as a colorless oil: ^1H NMR ($CDCl_3$, 250 MHz) δ 5.95-5.75 (m, 1H, RC*H*=CH_2), 5.03-4.90 (m, 2H, RCH=C*H*$_2$), 4.05 (m, 4H, CH_3CH_2OP), 2.02 (m, 2H, $CH_2CH=CH_2$), 1.80-1.20 (m, 20H); MALDI-FTMS (DHB) *m/z* 291.2088 ($C_{15}H_{31}O_3P + H^+$ requires 291.2089).

1-(Ethoxyhydroxyphosphinyl)-10-undecene (5). A solution of compound 4 (0.31 g, 1.07 mmol, 1.0 equiv) in CH_2Cl_2 (4.0 mL, 0.3 M) was treated dropwise with trimethylsilyl bromide (TMSBr, 0.17 mL, 1.28 mmol, 1.2 equiv). The reaction was stirred at 25 °C for 1 h, quenched with 5 mL of 5% [w/v] $KHSO_4$, and stirred vigorously for 5 minutes. The reaction mixture was then partitioned between ethyl acetate (100 mL) and water (100 mL), and the organic layer was washed with saturated aqueous NaCl (200 mL), dried (Na_2SO_4), and concentrated under reduced pressure. Chromatography (SiO_2, 2 x 8 cm, 12-20% CH_3OH-$CHCl_3$ with 1% aqueous NH_4OH) afforded 5 (0.10 g, 0.28 g theoretical, 36.2.%; most of the remaining mass was recovered as starting material) as a clear oil: ^1H NMR ($CDCl_3$, 250 MHz) δ 5.95-5.75 (m, 1H, RC*H*=CH_2), 5.03-4.90 (m, 2H, RCH=C*H*$_2$), 4.05 (m, 2H, CH_3CH_2OP), 2.02 (m, 2H, $CH_2CH=CH_2$), 1.80-1.20 (m, 20H). MALDI-FTMS (DHB) *m/z* 285.1589 ($C_{13}H_{27}O_3P + Na^+$ requires 285.1596).

10-(Ethoxyhydroxyphosphinyl)-decanoic acid (6). Compound 5 (0.10 g, 0.38 mmol, 1.0 equiv) in a biphasic solution composed of CCl_4-CH_3CN-H_2O (1.0 mL-1.0 mL-1.5 mL; total volume of 3.5 mL, 0.11 M) was treated sequentially with sodium periodate (0.31 g, 1.56 mmol, 4.1 equiv) and ruthenium trichloride hydrate (0.002 g, 0.009 mmol, 0.022 equiv). The reaction mixture was stirred at 25 °C for 2 h and then partitioned between CH_2Cl_2 (50 mL) and 1 N aqueous HCl (50 mL). The organic layer was washed with saturated aqueous NaCl (25 mL), dried (Na_2SO_4), and concentrated under reduced pressure. The resulting residue was resuspended in 40 mL of diethyl ether, filtered through a Celite pad, and concentrated under reduced pressure to afford 6 (0.09 g, 0.11 g

theoretical, 83%) as a colorless semisolid: ^{1}H NMR ($CDCl_3$, 250 MHz) δ 4.05 (m, 2H, CH_3CH_2OP), 2.32 (t, J = 7.5 Hz, 2H, CH_2COOH), 1.80-1.20 (m, 16H); FABHRMS (NBA-NaI) *m/z* 303.1340 ($C_{12}H_{25}O_5P + Na^+$ requires 303.1337).

FP-biotin, or 10-(fluoroethoxyphosphinyl)-*N*-(biotinamidopentyl)-decanamide (7). A solution of 6 (0.007 g, 0.025 mmol, 4.0 equiv) in CH_2Cl_2 (0.4 mL, 0.06 M) at -78 °C was treated dropwise with (diethylamino)sulfur trifluoride (DAST, 0.021 mL, 0.10 mmol, 4.0 equiv), brought to 25 °C, and stirred for 5 min. The reaction mixture was then treated with one-half reaction volume of dimethyl formamide containing *N*-hydroxysuccinimide (0.05 g, 0.25 mmol, 10 equiv) and stirred for an additional 10 min at 25 °C. The reaction mixture was then partitioned between ethyl acetate (50 mL) and water (50 mL), and the organic layer was washed with saturated aqueous NaCl (200 mL), dried (Na_2SO_4), and concentrated under reduced pressure to afford 10-(fluoroethoxyphosphinyl)-*N*-(hydroxysuccinyl)-decanamide (as judged by crude ^{1}H NMR; data not shown). Without further purification, this compound was treated with 5-(biotinamido)-pentylamine (Pierce, 0.0021 g, 0.062 mmol, 1.0 equiv) in MeOH (0.02 mL) and stirred for 10 min. The solvent was evaporated under a stream of gaseous nitrogen and the remaining residue was washed sequentially with diethyl ether and ethyl acetate, solubilized in a minimal volume of chloroform, transferred to a clean glass vial, and the solvent evaporated. This process was repeated once more to rid the desired biotinylated product of excess reagents and byproducts, affording 7 as a white film (0.0011 g, 0.0038 g theoretical, 29%): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.98 (b s, 1H, NH), 5.83 (b s, 1H, NH), 5.60 (b s, 1H, NH), 4.90 (b s, 1H, NH), 4.51 (m, 1H), 4.32 (m, 1H), 4.27 (m, 2H, CH_3CH_2OP), 3.22 (m, 4H, CH_2NHCOR), 3.15 (m, 1H), 2.92 (dd, J = 4.9 and 12.9 Hz, 1H), 2.72 (d, J = 12.9 Hz, 1H), 2.20 (m, 4H, CH_2CONHR), 1.85-1.24 (m, 31H); FABHRMS (NBA-NaI) *m/z* 593.3319 ($C_{27}FH_{50}N_4O_5PS + H^+$ requires 593.3302).

Preparation of Tissue Samples for Reaction with FP-Biotin. Rat tissues were Dounce-homogenized in Tris buffer (50 mM Tris-HCl buffer, pH 8.0, with 0.32 M sucrose). Tissue extracts were centrifuged sequentially at 1,100 x g (5 min), 22,000 x g (30 min), and 105,000 x g (60 min). The final supernatant (cytosolic fraction) was

adjusted to 1 mg protein/mL and then incubated for 30 min at 4 °C with one-tenth volume of avidin-agarose (Sigma) to deplete endogenous avidin-binding proteins. The resulting supernatant after a brief spin to pellet the avidin-beads (2 min at 10,000 x g) was removed and treated with FP-biotin as describe below.

Reaction of Protein Samples with FP-Biotin. Unless otherwise indicated, reactions between protein samples and FP-biotin were conducted as follows: FP-biotin (0.4 nmol) in $CHCl_3$ was added to a glass vial and the solvent evaporated under a stream of gaseous nitrogen. Ethanol (7.5 µL) was added to the vial, followed immediately by 192.5 µL of a 1 µg/µL protein stock in Tris buffer, and the reaction mixture was incubated at 25 °C for 30 min (final concentration of FP-biotin was 2 µM). The reaction mixture was quenched by adding one volume equivalent of standard 2X SDS-PAGE loading buffer (reducing) and heating the sample at 80 °C for 5 min. Reactions conducted for longer times (1 hr) or with higher concentrations of FP-biotin (20 µM) did not produce significant increases in the labeling intensity of most proteins, indicating that the majority of proteins had reacted to completion under the reported conditions. However, reactions with higher concentrations of FP-biotin did begin to show significant levels of nonspecific labeling (defined as the appearance of new protein bands that reacted with FP-biotin in both preheated and unheated samples).

Detection of FP-Biotin Reactive Proteins by SDS-PAGE-Western Blotting. Quenched FP-biotin reactions were run on SDS-PAGE (10 µg protein/gel lane) and transferred by electroblotting onto nitrocellulose membranes, which were blocked in TBS with 1% Tween (TBS-Tween) and 3% (w/v) nonfat dry milk for either 1 h at 25 °C or overnight at 4 °C. Blots were then treated with an avidin-horseradish peroxidase (HRP) conjugate (BioRad, 1:2000 dilution) in TBS-Tween with 1% nonfat dry milk for 30 min at 25 °C. The blot was washed with TBS-Tween three times (10 minutes/wash), treated with SuperSignal chemiluminescence reagents (Bio-Rad), and exposed to film for 0.1 to 8 minutes prior to development. For the comparison of STI-treated versus untreated Charotein samples, the relative amounts of FP-biotin labeling were estimated by film densitometry using an AlphaImager 2000 (AlphaInnotech).

Molecular Characterization of FP-Biotin Reactive Proteins. Brain soluble extracts were run over a Q sepharose column using an ÄKTA FPLC (Amersham Pharmacia Biotech) and eluted with a linear gradient of 0-500 mM NaCl. Samples of the elution fractions (10 x 2.5 mL fractions) were labeled with FP-biotin as described above, and those fractions containing the 75 kDa and 85 kDa labeled proteins were pooled and passed over a Mono-Q sepharose column. Proteins were eluted from the Mono-Q column with a linear gradient of 200-500 mM NaCl and those elution fractions enriched in the two labeled proteins were then run on SDS-PAGE and transferred to polyvinylidine difluoride (PVDF) membranes by electroblotting. Regions of the PVDF membranes containing the 75 and 85 kDa FP-biotin reactive proteins were excised, digested with trypsin, and the resulting peptides analyzed by matrix-assisted laser desorption ionization (MALDI) and MALDI-post-source decay time-of-flight mass spectrometry (Chaurand, et al. (1999) J. Am. Soc. Mass. Spectrom. 10, 91-103) on a Kratos Kompact Seq Instrument equipped with a curved field reflectron. The MALDI peptide data were used in MS-Fit and MS-Tag searches of the ProteinProspector databases (http://falcon.ludwig.ucl.ac.uk/mshome3.2.htm), which identified the 75 kDa protein as the rat orthologue of a human protein sequence KIAA0436 and the 85 kDa protein as acylpeptide hydrolase (APH).

Expression of Serine Hydrolases in HEK-293 Cells. The rat APH cDNA was cloned as follows. Primers were designed based on the enzyme's cDNA sequence (Kobayashi, et al. (1989) J. Biol. Chem. 264, 8892-8899)and used in polymerase chain reaction experiments to amplify a 1.4 kb partial cDNA clone from a rat liver 5' Stretch Plus cDNA library (Clontech). This amplified cDNA was used as a probe to isolate a full length APH cDNA from a liver library. The APH cDNA was subcloned into the eukaryotic expression vector, pcDNA3, and transiently transfected into HEK-293 cells as described previously. Transfected cells were harvested by trypsinization, washed with Hepes buffer (125 mM Hepes, pH 8.0, 100 mM NaCl) and Dounce-homogenized in Hepes buffer. Cytosolic and membrane fractions were isolated as described previously (Giang and Cravatt (1997) Proc. Natl. Acad. Sci. USA 94, 2238-2242) and labeled with FP-biotin as detailed above.

Design and Synthesis of a Biotinylated Fluorophosphonate, FP-Biotin. For the generation of a tagged, activity-based probe for the serine hydrolase family of enzymes, we considered several possible reactive groups and labeling strategies. Previous work by Glynn and colleagues had demonstrated that a saligenin phosphoramidate was a potent inhibitor of neuropathy target esterase (NTE) and could be synthesized with a biotin tag to identify this protein in tissue extracts (Glynn, et al. (1994) 301, 551-556). However, this inhibitor displayed remarkable specificity for NTE in these experiments, and thus appeared too selective to be useful as a general probe for serine hydrolases. Powers and colleagues had generated isocoumarin inhibitors coupled to biotin as serine hydrolase inhibitors (Kam, et al. (1993) Bioconjugate Chem. 4, 560-567; Winkler, et al. (1996) Mol. Immunol. 33, 516-623). While these isocoumarins reacted with a greater range of serine hydrolases than the aforementioned salgenin phosphoramidate, the requirement that these compounds alkylate a second functional group in the enzyme active site to achieve stable irreversible inhibition suggested that a significant number of serine hydrolases might remain insensitive to such reagents. In contrast, FP inhibitors seemed to satisfy the dual requirement of displaying 1) reactivity against the majority of serine hydrolases, and 2) selectivity for this enzyme family among the various classes of hydrolytic enzymes. While radiolabeled FPs were available commercially and through our own synthetic efforts (Patricelli, et al. (1999) Biochemistry 38, 9804-9812), the detection of such agents by fluorography requires several days to weeks (Patricelli, et al., *supra*; Keshavarz-Shokri, et al. (1999) Anal. Biochem. 267, 406-411), greatly limiting their general utility as rapid and high-sensitivity probes for profiling serine hydrolase expression and function.

10-undecen-1-ol (1)(numbering from Scheme 1) was converted to iodinated compound 3 through a tosylate intermediate (2). Reaction of 3 with excess triethylphosphite under reflux conditions afforded the diethoxy phosphonate 4, which was converted to the ethoxyhydroxy phosphonate 5 by treatment with trimethylsilylbromide (TMSBr). The double bond of 6 was oxidatively cleaved with ruthenium trichloride and sodium periodate (Carlsen, et al. (1981) J. Org. Chem. 46,

3k936-3938) to yield the terminal carboxylic acid product 6. Treatment of 6 with excess diethylaminosulfur trifluoride (DAST) and *N*-hydoxysuccinimide (NHS) afforded an *N*-succinyl fluorophosphonate intermediate which was reacted with 5-(biotinamido) pentylamine (NH_2-biotin) to generate FP-biotin (7). This synthetic route also allowed for the facile coupling of 6 to other reporter groups, including fluorescein cadaverine, which generated a fluorescent fluorophosphonate, FP-fluorescein [8; MALDI-FTMS (DHB) *m/z* 778.2671 ($C_{38}H_{47}FN_3O_8PS + Na^+$ requires 778.2703)].

FP-Biotin is an Activity-Based Probe for Serine Hydrolases. To initially test FP-biotin's utility as an activity-based probe for serine hydrolases, we reacted this agent with the mammalian serine amidase, fatty acid amide hydrolase (FAAH) (Cravatt, et al., (1996) Nature (London) 384k, 83-87). FP-biotin behaved as a potent irreversible inhibitor of FAAH (data not shown), displaying properties similar to those of other FP inhibitors of the enzyme (Patricelli, et al., *supra*; Deutsch, et al. (1997) Biochem. Pharmacol. 53, 255-260). We have shown that serine residue 241 serves as FAAH's catalytic nucleophile and mutation of this residue to alanine (S241A) generates an inactive enzyme (Patricelli, et al., *supra*). Therefore, FP-biotin (2 μM) was reacted with both FAAH and the S241A mutant (80 nM) for 10 minutes, after which the proteins were subjected to standard SDS-PAGE-Western blotting procedures using either anti-FAAH antibodies or avidin as detection reagents (Fig. 1A). While anti-FAAH antibodies identified both FAAH and the S241A mutant, avidin detected only FAAH in the FP-biotin reactions, demonstrating that this inhibitor exclusively reacted with the active form of the enzyme.

To further explore FP-biotin's reactivity with serine hydrolases, we incubated soluble fractions of rat testis with this inhibitor. Consistent with the abundance of proteases found in this tissue (Monsees, et al. ((1997) Adv. Exp. Med. Biol. 424, 111-123), FP-biotin labeled more than ten testicular proteins (Fig. 1B). Phosphonylated proteins of a variety of molecular masses were observed, ranging from 20-100 kDa, with a high concentration of labeled proteins found between 25-40 kDa, possibly representing members of the kallikrein clan of serine proteases (MacDonald, et al. (1996) J. Biol.

Chem. 271, 13864-13690). Importantly, boiling the protein sample prior to treatment with FP-biotin blocked nearly all protein labeling, further supporting that this tagged inhibitor reacts with serine hydrolases in an activity-dependent manner (Fig. 1B). Kinetic analyses revealed that the identified serine hydrolases displayed remarkably different rates of FP-biotin reactivity, with two of the larger proteins labeling to apparent completion within one minute (Fig. 1C, top panel) and most of the smaller proteins reacting more slowly over the course of several minutes (Fig. 1C, bottom panel).

Considering that many serine proteases exist *in vivo* as inactive complexes with endogenous inhibitory proteins (Kato (1999) Hum Mutat. 13, 87-98; Declerck, et al. (1997) Adv. Exp. Med. Biol. 425, 89-97; Monsees, et al., *supra*), we compared the ability of FP-biotin to react with both free and inhibitor-bound proteases. While FP-biotin reacted strongly with free trypsin, the tagged inhibitor did not label a trypsin sample that was preincubated with the Kunitz-type serine protease inhibitor, soybean trypsin inhibitor (STI), despite the presence in the latter reaction of significantly greater amounts of trypsin (Figure 1D, left panel). Soluble fractions of rat testis were also exposed to STI and then treated with FP-biotin. Consistent with the relatively broad specificity of this protease inhibitor, several, but not all FP-biotin reactive proteins showed significantly lower labeling intensities in the STI-treated sample (Figure 1D, right panel).

Collectively, these results highlight that FP-biotin can detect differences in the functional state of a serine hydrolase, even in the special cases where enzyme activity varies without correlation to enzyme quantity. Such observations gain particular significance when one considers the complexity and diversity of serine proteases and inhibitors typically present in whole cell and tissue samples (Kato, *supra*; Declerck, *supra*; Deutsch, et al., *supra*). Without an activity-based probe like FP-biotin, standard genomics and/or proteomics studies would have difficulty distinguishing free (active) from inhibitor-bound (inactive) proteases in these samples. Finally, the ability to monitor rates of FP-biotin labeling can greatly assist in the identification of even quite subtle changes in serine hydrolase activities.

Molecular Characterization of FP-Biotin Reactive Proteins. In order to verify that the proteins labeled by FP-biotin in crude tissue extracts were indeed serine hydrolases, two phosphonylated proteins were isolated from rat brain cytosol. The most strongly labeled brain proteins ranged from 75-85 kDa in size (Fig. 2A) and eluted from a Q sepharose column between 300 and 450 mM NaCl (Fig. 2B). To estimate the abundance of these proteins in the Q elutions, the intensity of their labeling with FP-biotin was compared to that of a serial dilution of a FAAH sample reacted to completion with the inhibitor (Fig. 2C). Both the 75 and 85 kDa FP-biotin reactive proteins displayed labeling intensities similar to that of a 20 ng sample of FAAH (0.35 pmoles), setting a lower limit for the quantity of these proteins that was well within the range needed to obtain protein sequence information. The 85 and 75 kDa proteins were identified by standard protein chemistry techniques as acylpeptide hydrolase (APH) (Kobayashi, et al. (1989) J. Biol. Chem. 264, 8892-8899), a serine peptidase that has been shown to react with DIFP (Scaloni, et al. (1992) J. Biol. Chem. 267, 3811-3818), and the rat orthologue of a human protein sequence KIAA0436 (Ishikawa, et al. (1997) DNA Res. 4, 307-313). Interestingly, a homology search revealed that the KIAA0436 protein shares 30% identity with the prokaryotic enzyme Protease II, also an established serine hydrolase that reacts with DIFP (Yoshimoto, et al. (1995) J. Biochem. 117, 654-660). The Ser-His-Asp catalytic triad residues of Protease II were conserved in the KIAA0436 protein, supporting that this mammalian protein is a novel member of the protease II family of serine proteases. Finally, FP-biotin also labeled a 100 kDa brain protein that appeared to be expressed at much lower levels (equivalent to 15 fmoles, or ~ 1 ng, of FAAH), demonstrating that this tagged inhibitor can readily detect subnanomolar concentrations of serine hydrolases (15 fmol/20 μL per gel lane).

To test whether FP-biotin could record changes in the expression level of serine hydrolases in crude cellular extracts, we transfected cDNAs for both APH and FAAH into HEK-293 cells. Treatment of the cytosolic and membrane fractions of these cells with FP-biotin identified a strongly phosphonylated 85 kDa protein in the APH-transfected cells (Figure 3A and B; I), but not in control cells transfected with either empty vector or the FAAH cDNA. In contrast to this labeling pattern, an abundant 65

kDa phosphonylated protein was identified exclusively in the membrane fraction of FAAH-transfected cells (Figure 3B; II), consistent with previous characterizations of this serine hydrolase as an integral membrane protein (Giang and Cravatt, *supra*, Patricelli, et al. (1998) Biochemistry 37, 15177-15187). Longer exposures of the cytosol blot identified in the mock and FAAH-transfected HEK cells a weak 85 kDa signal that may represent endogenous levels of APH in this cell type (Figure 3C; I).

In summary, the data presented in Figures 1-3 demonstrate that FP-biotin can: 1) react with numerous serine hydrolases in crude cell and tissue samples, 2) detect subnanomolar concentrations of serine hydrolases, and 3) record differences in both the functional state and expression level of these enzymes. It is also important to highlight that the identification of FP-biotin labeled proteins using standard avidin-HRP chemiluminescence assays is extremely rapid (requiring exposure times of only seconds to minutes), making this chemical agent particularly well suited for high throughput proteomics investigations. Additionally, the covalent attachment of a biotin molecule to phosphonylated serine hydrolases should assist in the subsequent biochemical characterization of these enzymes. For example, Schriemer and colleagues have recently developed a method that combines immobilized avidin beads with MALDI mass spectrometry to facilitate the chemical analysis of biotinylated proteins and peptides (Schreimer, et al. (1998) Anal. Chem. 70, 1569-1575). If integrated with FP-biotin, this technique allows for the molecular identification of serine hydrolases (as well as their respective catalytic nucleophiles) directly from whole cell and tissue samples.

Profiling Serine Hydrolases in Rat Tissues with FP-Biotin. To test FP-biotin's ability to resolve complex patterns of serine hydrolase expression, we compared the profiles of phosphonylated proteins from soluble extracts of rat brain, liver, testis, and prostate (Figure 4). In the lower molecular mass range, clear tissue-specific and tissue-restricted FP-biotin reactive proteins were identified (Figure 4A). Interestingly, a strongly labeled 33 kDa protein was identified exclusively in prostate (III). While the molecular size of this phosophonylated protein is consistent in mass with human prostate specific antigen (PSA) (Bei, et al. (1995) J. Clin. Lab. Anal. 9, 261-268), a serine protease

expressed primarily in this tissue, orthologues of PSA are not thought to exist in rodents based on previous molecular (Southern blots) and cell biological (immunocytochemistry) studies (Karr, et al. (1995) Cancer Res. 55, 2455-2462). The identification of an FP-biotin reactive protein abundantly and selectively expressed in rat prostate suggests that this organism may indeed possess functional (but not necessarily high sequence-related) homologues of human PSA, an observation that merits further investigation considering PSA's status as a principal marker for prostate cancer (Polascik, et al. (1999) J. Urol. 162, 293-306). Several other FP-biotin reactive proteins also displayed tissue-restricted patterns of expression, including a testis-specific 42 kDa protein (I) and two 38 kDa proteins, one of which was found in brain and testis, and the other in brain and liver (II). In the larger molecular mass range, most of the FP-biotin reactive proteins appeared to display broad tissue distributions (Figure 4B). However, a labeled 65 kDa protein was found in highest relative abundance in liver, at lower levels in testis and prostate, and was not detected in brain (I). Similarly, a phosphonylated 70 kDa protein was found exclusively in liver (II).

It is evident from the above results that the subject compositions and methods provide for identification of serine hydrolases, as related to mixtures of serine hydrolases from cells, tissue or the like, discrimination between cells having different serine hydrolase activity profiles, where the cells may differ between normal and neoplastic, state or nature of differentiation, source, and the like. By using the subject compositions, the aberrant state of tissue or cells may be determined in relation to the serine hydrolases being expressed, the serine hydrolase activity of the composition, the change in serine hydrolase activity in relation to changes in the environment of the sample, e.g. chemical or physical change, etc. The subject compositions allow for identification and isolation of serine hydrolases, which allows for their characterization and a determination of their role

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for determining serine hydrolase activity in a sample suspected of having at least one serine hydrolase, said method comprising:

combining said sample with a compound comprising a fluorophosphoric acid derivative bonded through a linking group to a ligand;

separating any serine hydrolase bound compound; and

detecting said serine hydrolase bound compound by said ligand;

whereby said serine hydrolase activity in said sample is determined.

2. A method according to Claim 1, wherein said compound is a fluorophosphonate and said linking group comprises an alkylene of at least 2 carbon atoms.

3. A method according to Claim 1, wherein said ligand is biotin, and labeled strept/avidin is bound to said biotin.

4. A method according to Claim 1, wherein said ligand is fluorescein.

5. A method for determining serine hydrolase activity in a physiological sample suspected of having at least one serine hydrolase, said method comprising:

combining said sample with a compound comprising a fluorophosphonate derivative bonded through a linking group comprising an alkylene of at least 2 carbon atoms to a ligand to provide a reaction mixture; and

detecting said serine hydrolase compound bound by said ligand;

whereby said serine hydrolase activity in said sample is determined.

6. A method according to Claim 5, wherein said sample is a cellular lysate.

7. A method according to Claim 5, wherein said detecting is taken at different times with aliquots of said reaction mixture.

8. A method according to Claim 5, wherein said detecting comprises separating serine hydrolase bound by said ligand from other components in said reaction mixture by means of electrophoresis.

9. A method according to Claim 9, wherein said detecting comprises binding said ligand to a labeled receptor for said ligand.

10. A method according to Claim 9, wherein said ligand is a fluorescer and said detecting comprises detecting fluorescence of said serine hydrolase bound by said ligand.

11. A method according to Claim 5, comprising the further step of identifying serine hydrolases present in said sample by means of monoclonal antibodies specific for at least one serine hydrolase.

12. A method for discovering unknown serine hydrolases, said method comprising:

combining a biological source of proteins with a fluorophosphonate derivative bonded through a linking group comprising an alkylene of at least 2 carbon atoms to a ligand to provide a reaction mixture;

isolating said serine hydrolase compound bound by said ligand; and

comparing at least one characteristic of said ligand bound serine hydrolase with know serine hydrolases, whereby said ligand bound serine hydrolase can be determined to be a novel serine hydrolase.

13. A method according to Claim 12, wherein said characteristic is at least a partial amino acid sequence.

14. A compound of the formula:

$$X - L - R - (O)_m - P(O)(OT) - (F \text{ or } SH)$$

wherein:

F, P and O have their normal meaning of fluoro, phospho and oxy;

X is a ligand or detectable label;

L is a linking group;

R is an aliphatic group of at least 2 carbon atoms and not more than about 16 carbon atoms;

SH is a serine hydrolase bonded at serine of the active site to the phosphorous;

m is 0 or 1; and

T is alkyl of from 1 to 6 carbon atoms.

15. A compound according to Claim 14, wherein L is an dicarboxamido-α, Ω-alkylene, wherein said alkylene is of from 2 to 6 carbon atoms.

16. A compound according to Claim 14, wherein said ligand is biotin.

17. A compound according to Clam 14, wherein said detectable label is fluorescein.

18. A compound according to Claim 14, wherein F is present, L is dicarboxamido-α, Ω-alkylene, wherein said alkylene is of from 2 to 6 carbon atoms, and R is an alkylene group.

19. A method for diagnosing an indication in relation to serine hydrolase activity associated with said indication, said method comprising:

obtaining a sample from a source suspected of having said indication

combining said sample with a compound comprising a fluorophosphoric acid derivative bonded through a linking group to a ligand to provide a reaction mixture;

determining a change in said serine hydrolase activity of said reaction mixture or a change in the amount of protein bound to said compound; and

comparing said serine hydrolase activity or amount with a serine hydrolase standard;

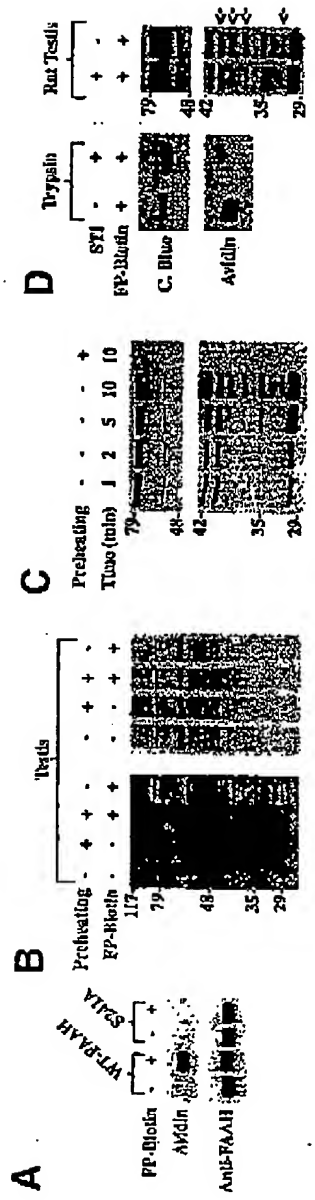
whereby said indication is diagnosed.

20. A method according to Claim 19, wherein said fluorophosphoric acid derivative is a compound according to Claim 14.

METHODS AND COMPOSITIONS FOR ANALYZING FOR SERINE HYDROLASES

ABSTRACT OF THE DISCLOSURE

Derivatized fluorophosphoric acid compounds are used as irreversible inhibitors of serine hydrolases. The derivatives have a ligand, which allows for isolation and detection of the inhibited serine hydrolases. The derivatized fluorophosphoric acid compounds react with active serine hydrolases and are useful in determining serine hydrolase activity of a sample, react at different rates with different serine hydrolases, so as to allow for differentiation and analysis of samples having a plurality of serine hydrolases, and can be used in identifying cellular sources, disease indications, and the like.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Avidin
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HT-PAAH
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C
Preheating
Time (min)
79
48
42
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D
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SFI
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Trypsin
C. Blue
Avidin
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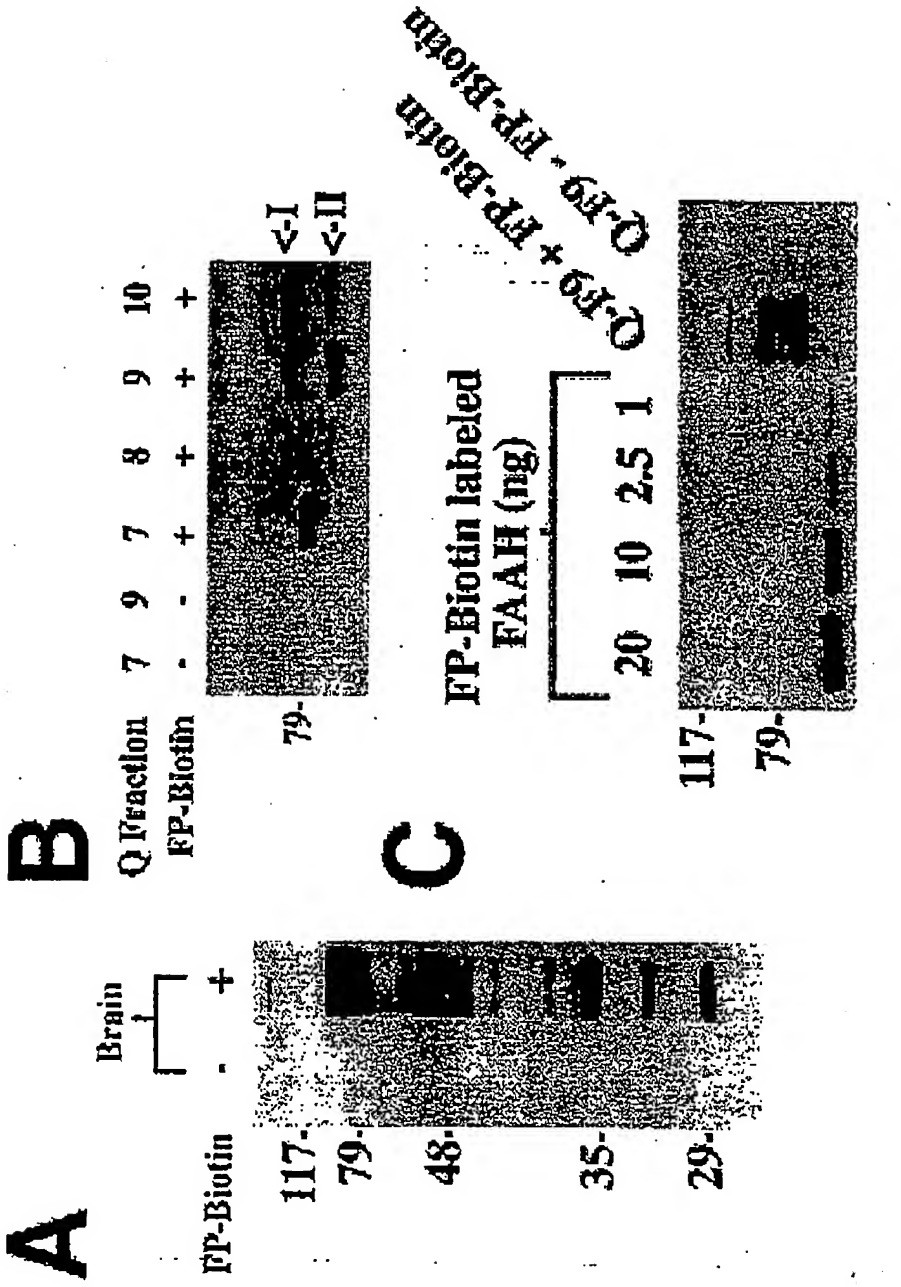
35
29

FIGURE 1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Brain
FP-Biotin - +
117-
79-
48-
35-
29-
B
Q Fraction 7 9 7 8 9 10
FP-Biotin - - + + + +
79-
I->
II->
C
FP-Biotin labeled FAAH (ng)
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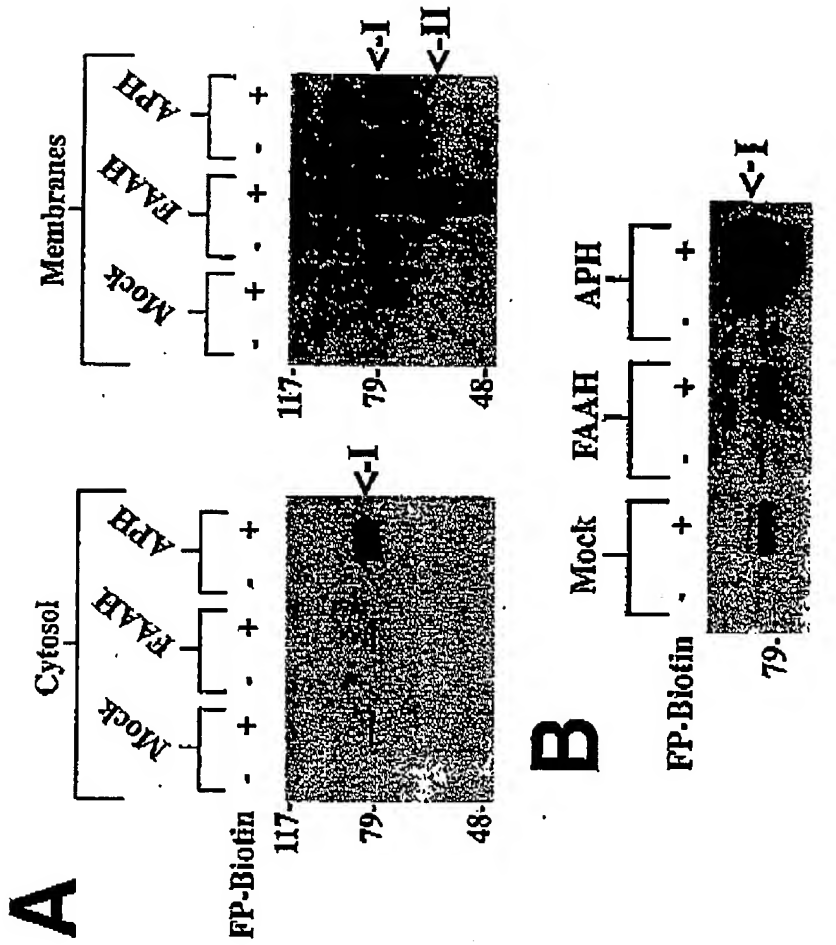
Q-Tg + FP-Biotin
Q-Tg - FP-Biotin
117-
79-

FIGURE 2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Cytosol
Membranes
Mock
EAAH
APH
FP-Biotin
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<-II
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79-
48-
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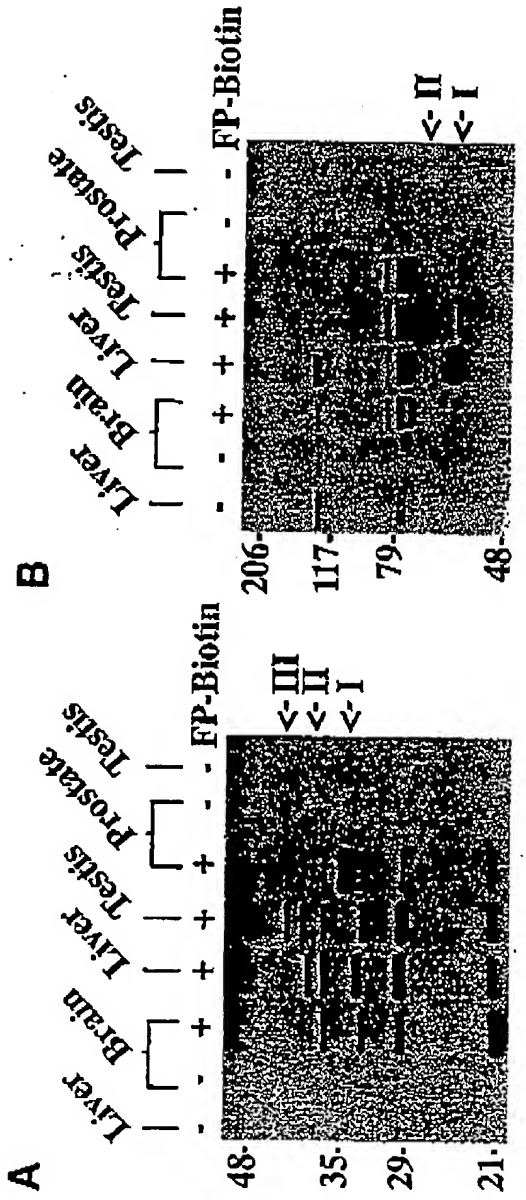
Mock
EAAH
APH
FP-Biotin
<-I
79-

FIGURE 3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Brain
Liver
Testis
Prostate
Testis
FP-Biotin
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<- II
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35
29
21
B
Liver
Brain
Liver
Testis
Prostate
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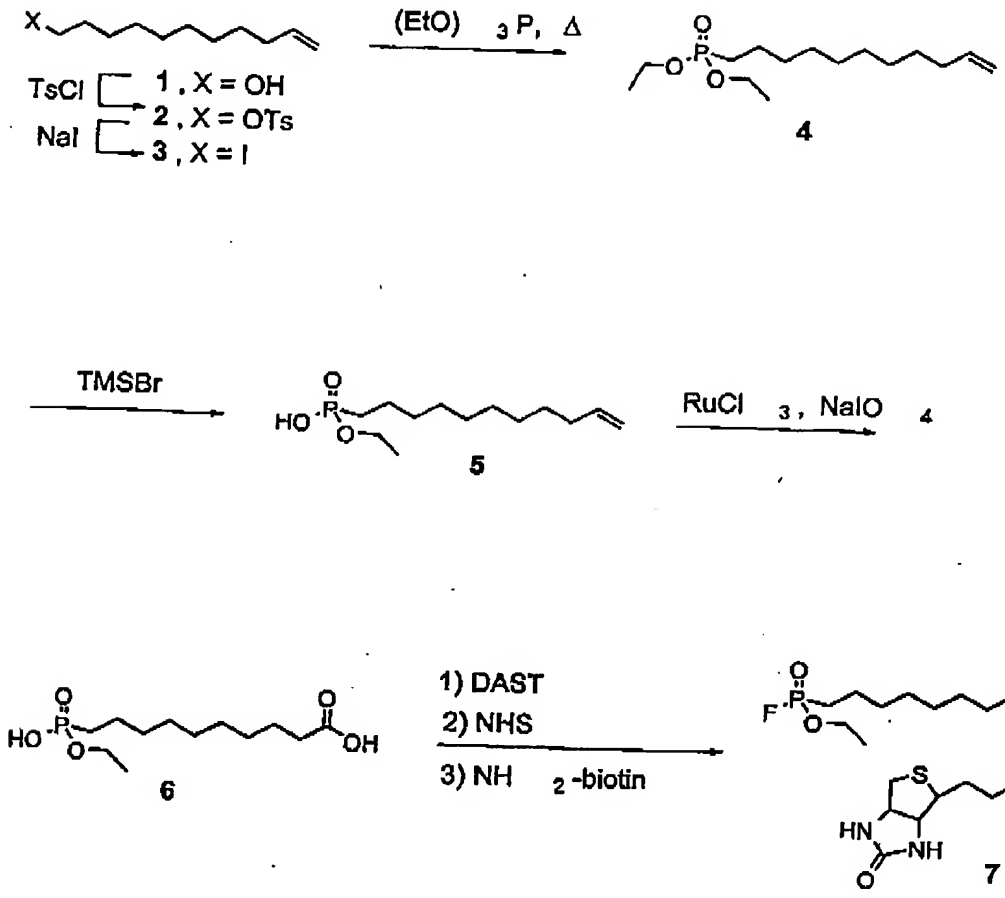
FP-Biotin
<- II
<- I
206
117
79
48

FIGURE 4

FIGURE 5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NaI
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2 , X = OTs
3 , X = I
(EtO) 3 P, Δ
4
TMSBr
5
RuCl 3 , NaIO 4
6
1) DAST
2) NHS
3) NH 2 -biotin
7 , FP-biotin
8 , FP-fluorescein